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14. ABSTRACT

Hormone replacement therapy (HRT) may increase the risk of breast cancer (BC) in post-menopausal women. Due to cessation of menstrual blood, an increased iron level is one of the most important physiological changes in post-menopausal women. We postulate that HRT together with high iron in post-menopausal women may lead to increased risk of BC. We have tested this hypothesis in cell culture models with different status of estrogen and progesterone receptors as well as an iron loaded transgenic mouse model. Our results have shown that Prempro, the most prescribed HRT drug, have induced cell proliferation only in estrogen receptor positive cells and estrogen is the main active component in the drug for the proliferation. Estrogen also induced transferrin receptor, a membrane protein controlling iron uptake. In animal study, female wild type and iron overloaded HFE homozygote mice were inoculated with mouse mammary cancer cells into the fat pads and then fed a diet with or without Prempro. No significant differences in tumor incidence were observed among mice. In conclusion, our hypothesis was confirmed in an in vitro tissue culture system. However, animal study failed to produce mammary tumors in our proposed iron overloaded transgenic mice.

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Hormone replacement therapy, iron, estrogen, cell proliferation, progesterone, breast cancer

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Introduction:

Menopause is a natural aging process during which time a woman passes from the reproductive to the non-reproductive years. Through a complex system of hypothalamic-pituitary-ovarian (HPO) glands, the pituitary gland stimulates the ovaries to mature and release an egg every month. Estrogen and progesterone together help regulate this event. As a woman matures, the ovaries have fewer eggs to stimulate, and eventually estrogen and progesterone production in the ovaries ceases. Many women experience physical changes during and after menopause. One of the first physiological changes is in menstrual periods. During the natural menopause transition, the dynamics of the HPO hormones change dramatically from cyclic to static patterns and levels of estrogen and progesterone from high to low. Many post-menopausal women may experience patho-physiological conditions or menopause symptoms such as "hot flashes" or night sweats, osteoporosis, and thinning and drying of skin.

To ease menopausal symptoms and prevent osteoporosis, a large number of post-menopausal women in the United States use estrogen-based hormone replacement therapy (HRT). Recent epidemiological studies from the Women's Health Initiative indicate that HRT may actually increase the risk of breast cancer (BC), as well as heart attacks, strokes, and blood clots (*1*). A meta-analysis of nine prospective studies on post-menopausal levels of endogenous sex hormones and BC showed a strong association of estrogens with BC risk (*2*), a rate which is much higher in post-menopausal than pre-menopausal women. Yet, serum circulating estrogen levels are lower in post-menopausal than in pre-menopausal women and breast tissue estrogen levels are comparable between the two groups. How a low level of estrogen contributes to a high BC incidence rate in post-menopausal women is an important research topic, which has not yet been addressed or studied. In this dilemma, a re-evaluation of the risk factors for BC is necessary. Because of the female baby-boomer generation entering this menopausal status, now there is an even greater need to address BC etiology associated with HRT or estrogens.

In the present study, we have hypothesized that, due to the cessation of menstrual bleeding, a high iron level is one of the pre-neoplastic changes in post-menopausal women. HRT or estrogen together with iron increase BC risk through mechanisms of increased cell proliferation or oxidative stress. This hypothesis was tested in cell culture model systems and in an iron loaded transgenic mouse model. Since iron slowly accumulates due to the mutation of the HFE gene (hemochromatosis Fe), iron elevated in the mouse body mimics the post-menopausal condition.

Development of iron overloaded transgenic mice:

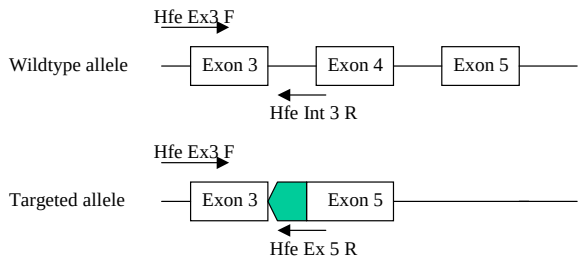

Figure 1. HFE gene knockout.

The murine HFE gene is structurally similar to the human gene. Four different HFE gene disruptions have been reported in the mouse: an exon 4 knockout, an exon 3 disruption/exon 4 knockout, an exon 2-3 knockout, and a C282Y knock-in (*3-5*). In each model, the mice manifest increased hepatic iron levels, elevated transferrin saturation, and increased intestinal iron absorption. These mice also demonstrate relative sparing of iron loading in reticuloendothelial cells. We have used the exon 4 knockout

mice provided by Dr. Nancy Andrews of the Howard Hughes Medical Institute, Harvard Medical School, which are currently bred in our laboratory (*4*). Figure 1 shows the wild type allele and the targeted allele of the HFE knockout mice. Using RT-PCR with primers of HFE Ex3 F: **5'** GTCACGAA GTTGGGA GTGGT **3',** HFE Int3 R: **5'** CAGCCTTGGCTACAGTGTGA **3'** and HFE Ex5 R: **5'** ATGGTGACCCC ACTG ATGAT **3',** the designed PCR products of the following two primer sets of HFE Ex3 F/ HFE Int 3 R will be about 500 bp (wild-type) and HFE Ex3 F/ HFE Ex5 R will be 400 bp (HFE homozygote).

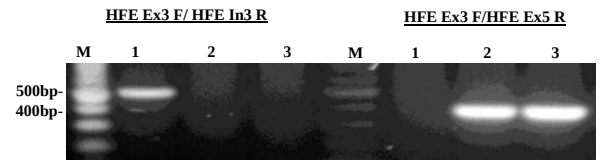

Figure 2. Genotyping of HFE mice. M: marker; 1. Wild type; 2. HFE homozygote; 3. first offspring HFE homozygote.

Genotype of HFE mice by RT-PCR:

Figure 2 shows the genotyping of the wild-type C57BL6 mouse, the original HFE homozygote, and the first offspring of HFE homozygote mice. The left panel of Figure 2 shows a band of 500 bp (primer set HFE Ex3 F/HFE Int 3 R) by the wild-type but not by HFE homozygotes. The right panel shows a band of 400 bp by HFE homozygotes but not by the wild-type mouse. It is expected that HFE heterozygotes will produce double bands (400 bp and 500 bp), though $HFE^{+/-}$ mice have not yet been bred in the laboratory and were not used in the present study.

Evaluation of iron status in sera of wild type and iron overload HFE transgenic mice:

Serum iron (SI), serum unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), and transferrin saturation (TS, %) are determined as follows: At an acidic pH (pH 4.5) and in the presence of hydroxylamine (a reducing agent), transferrin-bound iron dissociates to release ferrous ions. These react with ferrozine to form a stable magenta-colored complex (Fe^{2+}-ferrozine) with a maximum absorption at 560 nm. The difference in absorbance at 560 nm before and after ferrozine addition in the serum sample is proportional to SI concentration. In contrast to SI, serum UIBC is measured at alkaline pH (TRIZMA®, pH 8.1). Ferrous ions added to the serum bind specifically to transferrin at unsaturated iron-binding sites and then the remaining unbound ferrous ions are measured with the ferrozine reaction. The difference between the amount of unbound iron and the total amount added to serum is equivalent to the quantity bound to transferrin, which is the UIBC. The serum TIBC equals the SI plus the UIBC. Serum transferrin saturation (%) is calculated using (SI divided by TIBC) x 100.

Table 1. SI and TS levels in wild type and HFE homozygote mice

	SI (μM)	TS (%)
Wild-type	28.6	39.1
Homozygotes	88.1	91.8

Table 1 shows the levels of serum iron (SI) and transferrin saturation (TS) in a ten-week-old wild-type C57BL6 and the first offspring of $HFE^{-/-}$ mouse at the same age. The differences in iron levels between the two mice types were striking, even though these mice were fed with the Purina base diet without iron supplementation.

Characterization of estrogen receptor status in two mouse mammary cancer cell lines:

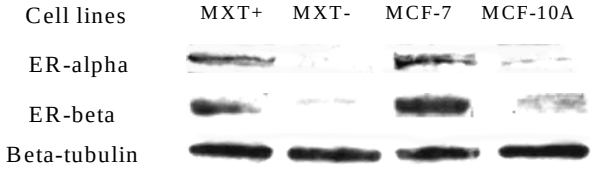

Figure 3. Western blot analysis of ER protein. Beta-tubulin was used as an internal control for protein loading. ER-beta loading is much more than ER-alpha.

MXT^+ (estrogen receptor positive and progesterone receptor positive; ER^+/PR^+) and MXT^- (ER^-/PR^+) cells were gifts from Dr. G. Bernhardt of the Institute of Pharmacy, University of Rogensburg, Germany, and have been available in our laboratory (*6*). The MXT^+ cell line was derived from the murine mammary cancer model MXT^-M-3,2 MC (hormone sensitive), which was first induced by urethane treatment in female C57BLxDBAfF1 mice as previously described (*7*). MXT^- cell line was derived from the MXT-M-3,2 (OVX) MC (hormone insensitive) mice (*8*). Figure 3 shows the ER status of both cell lines. They were characterized in our lab by Western blot along with the human breast cancer cell lines MCF-7, known as ER^+, and the immortalized human breast epithelial cell line MCF-10A, known as ER^-. Because MXT^+ cells contain PR, these results indicate that ER in the MXT^+ cells (ER^+/PR^+) is functional.

Changes in study design:

As originally proposed in this award, we intended to inoculate cells from the human breast cancer cell line MCF-7 into the mammary fat pads of our transgenic mouse model. However, this is not a valid approach because human cancer cells will only grow in nude mice but not in the iron overloaded transgenic mice that we have been using. We decide to use the mammary cancer cell line MXT^+ (ER^+ and PR^+) for the present study.

Injection of mammary cancer cells into the fat pads of various types of mice:

HFE transgenic mice with a mixed C57BL/6J X 129/SvEvTac background were injected subcutaneously into the right and left inguinal (mammary fat pad) area with 1 x 10^6 MXT^+ cells. No mammary tumors were observed for 6 months. These results indicate that MXT^+ cells may be rejected by the HFE mice because of the genetic difference.

To further test whether the genetic difference plays a role, twenty B6D2F1/Taconic female mice were divided into five groups fed with different diets based on Purina. Five groups were as follows: (1) HRT + iron diet (50 mg Prempro^{TM}/kg Purina + 1% w/w ferrous sulfate); (2) 1x10^6 MXT^+ cells injected with HRT + iron diet; (3) 1x10^6 MXT^+ cells with control diet; (4) 1x10^6 MXT^- cells with HRT + iron diet; (5) 1x10^6 MXT^- cells with control diet. No significant differences in weight were observed among the five groups, suggesting that the diet mixed with iron and Prempro^{TM} did not induce toxicity. Mammary tumors appeared 78 $\pm$ 16 days after MXT^+ cell injection and 183 $\pm$57 days after MXT^- cell injection. No significant differences in tumor latency were observed between the control diet and diet enriched with Prempro^{TM} and iron. These results suggest that MXT cells can grow mammary tumors in mice with the same genetic background.

When MXT^+ and MXT^- cells were injected into C57BL/6Nac/Taconic or 129/SvEvTac female mice, no mammary tumors appeared. However, the injection of MXT cells into nude mice developed mammary tumors. These results indicate that the MXT cells are potent in

inducing mammary tumors in its own strain or immune deficient mice but not in C57BL or HFE with C57BL and 129/SvEvTac mice.

Because of these data, we decided to inject 4T1 mouse mammary cancer cells, which are much more potent than MXT cells. 4T1 cells are estrogen receptor negative (ER-) and were originated from BALBc mice. Our HFE mice are a mixed background of C57BL/6 and 129/SvEvTac with a brown color indicating more of 129 background. The following results showed that 4T1 cells initially induced mammary tumors but disappeared 3 weeks after injection.

	HFE mice (5 mice)
Day 10	100% had tumors
Day 17	70% had tumors
Day 20	0% had tumors

	129 mice (5 mice)
Day 8	100% had tumors
Day 13	78% had tumors
Day 15	20% had tumors
Day 22	0% had tumors

	C57 mice (5 mice)
Day 8	95% had tumors
Day 13	30% had tumors
Day 15	30% had tumors
Day 22	10% had tumors
Day 25	0% had tumors

Based on these data, we conclude that MXT cells and 4T1 cells are not capable of inducing mammary tumors in mice with C57 and/or 129 genetic backgrounds, including our proposed HFE mice. Since mouse mammary cancer cell lines from C57 or 129 background mice do not exist or are not commercial available, we concluded that our proposed *in vivo* study was not feasible. Therefore, we have used our tissue culture models to test the proposed hypothesis that HRT or estrogen together with iron increase BC risk through mechanisms of increased cell proliferation.

Additive or synergistic effects of E2 and iron on cell proliferation (see Appendices):

In the following study, we have investigated whether a concomitant exposure to estrogen and iron synergistically enhance *in vitro* proliferation of various cell types with different status of estrogen receptor (ER) and progesterone receptor (PR). Human breast cancer cell line, MCF-7 (ER^+) and non-cancerous breast epithelial cell line MCF-10A (ER^-), as well as MXT^+ (ER^+/PR^+) and MXT^- (ER^-/PR^+) cells were used. Crystal violet assay was employed to determine cell proliferation. Our results have shown that Prempro^{TM} has induced cell proliferation only in ER^+ cells. For example, at 10 $\mu g/cm^2$ of dose, Prempro-treated MCF-7 cell proliferation was 2.3-fold of controls in α-MEM completed with 10% fetal bovine serum (FBS). To further distinguish the effects of estradiol (E2) and progesterone (Pg) on cell proliferation, MCF-7 cells were separately treated with E2 ($1x10^{-9}$ M) or Pg ($4x10^{-9}$ M). Results showed that E2 is the main component inducing cell proliferation. MCF-7 cells were then treated with E2 and/or iron in α-MEM

medium containing 0.1% FBS (to minimize transferrin iron in serum, a confounding factor). Our results showed that E2 together with iron induced higher cell proliferation (15.3-fold of control) than either E_2 (6.1-fold) or iron (7.9-fold) alone. Western blotting of E2-treated cells showed that E_2 also induced transferrin receptor (TfR), a membrane protein involved in cellular iron uptake. In conclusion, our results suggest that iron and estrogen may be concomitantly involved in the cell proliferation of ER^+ breast cells and together may contribute to the increased risk of breast cancer in post-menopausal women (detailed data are included in the Appendices).

Key Research Accomplishments:

1) Bred and characterized the iron overloaded transgenic mice in our laboratory.

2) Evaluated the iron status in sera of wild type and HFE transgenic mice, validating our model mimicking post-menopausal conditions. Due to the cessation of menstrual cycling, iron levels in post-menopausal women are higher than in pre-menopausal women.

3) Assessed the presence of the estrogen receptor and progesterone receptor status of different human breast cancer and murine mammary cancer cell lines. Hormone replacement therapy (HRT) mainly consists of estrogen and progestin, hence, knowing the receptor status is important for data interpretation.

4) Injected MXT cells as well as 4T1 cells into different strains of mice. The proposed iron overload HFE mice were generated from C57BL/6J and 129/SvEvTac mice. Although these two cell lines of MXT and 4T1 induced mammary tumors in their own strains B6D2F1 or BALBc, they were not able to induce mammary tumors in C57 or 129 or the mixed background HFE mice.

5) Showed the proliferating effects of Prompro^{TM}, the most prescribed drug for HRT, only in ER^+ cells but not in ER^- cells. Estrogen was the main active component in Prempro^{TM} in inducing cell proliferation.

6) Demonstrated *in vitro* the additive or synergistic effects of iron and estrogen on cell proliferation.

7) Most importantly, elucidated one of the mechanisms of the interaction of estrogen with iron. E2 induces transferrin receptor (TfR), a membrane protein controlling cellular iron uptake.

Reportable Outcomes:

Publication:

Jisen Dai, Maarten Bosland, Lori Horton, Krystyna Frenkel, Günther Bernhardt, and **Xi Huang*** "Enhancement of cell proliferation by iron and estrogen in breast epithelial cells with different status of estrogen and progesterone receptors– A role for iron in breast cancer of post-menopausal women?" Submitted.

Abstracts:

Dai, J. S., and **Huang, X.** 96th Annual Meeting of the American Association for Cancer Research, April 16-20, 2005, Anaheim, CA. "Role of hormone replacement therapy in breast cancer cell proliferation." Proceedings CD-ROM, volume 46.

Eckard, J., Frenkel, K., **Huang, X.** 96th Annual Meeting of the American Association for Cancer Research, April 16-20, 2005, Anaheim, CA. "Iron metabolism and oxygen tension: effects on hypoxia-inducible factor (HIF) stabilization." Proceedings CD-ROM, volume 46.

Personnel:

Xi Huang, Ph.D. Principal Investigator, 10% effort

Jisen Dai, M.D. Research Associate, 50% effort.

Conclusions:

With the funding support of this concept award, we have performed the feasibility study of growing various murine mammary cancer cells with different estrogen receptor status in our iron loaded transgenic mouse model. Although we had difficulty in carrying out our proposed *in vivo* study due to the genetic differences among cell lines and mice, we have shifted our research to *in vitro* systems. Our *in vitro* results suggest that iron and estrogen may be concomitantly involved in the cell proliferation of ER^+ breast cells and that together may contribute to the increased risk of breast cancer in post-menopausal women.

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Appendices: 3

2094 Role of hormone replacement therapy in breast cancer cell proliferation

Jisen Dai and Xi Huang. *New York University School of Medicine, New York, NY.*

An estimated 10 million post-menopausal women in the United States use estrogen-based hormone replacement therapy (HRT) to ease menopausal symptoms and prevent osteoporosis. Epidemiological studies from the Women's Health Initiative (WHI) indicate that HRT may actually increase the risk of breast cancer (BC). However, whether a causative relationship exists between HRT and BC remains unclear. In the present study, we have investigated the effects of HRT on proliferation of various BC cells with different estrogen receptor (ER) status. Human BC cell line, MCF-7 (ER^+) and non-cancerous breast epithelial cell line MCF-10A (ER^-), as well as two murine mammary cancer cell lines, one MXT^+ [ER^+ and progesterone receptor positive (PR^+)] and the other MXT^- (ER^- but PR^+) were used. Crystal violet assay was employed to determine cell proliferation. Our results have shown that Prempro^{TM} (Wyeth-Ayerst Pharmaceuticals), the most prescribed HRT drug consisting of estrogen (E2) and progesterone, have induced cell proliferation in ER^+ cells. For example, at 10 μg/cm^2 of dose, Prempro-treated MCF-7 cell proliferation was 2.3-fold of control in α-MEM completed with 10% fetal bovine serum (FBS). The same dose in MXT^+ cells induced 1.7-fold of control of cell proliferation. For the concentrations tested (1, 2, 5, and 10 μg/cm^2), it increased in a dose-dependent manner. In ER^- cell lines of MCF-10A and MXT^-, Prempro^{TM} didn't show any effects on cell proliferation. To further distinguish the effects of E2 and progesterone on cell proliferation, MCF-7 cells were separately treated with E2 ($1x10^{-9}$ M) or progesterone ($5x10^{-9}$ M). We have shown that E2 is the main component in inducing cell proliferation and progesterone has no effects. Tamoxifen, an E2 antagonist targeting ER, can inhibit the proliferating effects of E2. Because HRT is mainly used in post-menopausal women and this population generally has a higher level of iron due to the cessation of menstrual blood, we have further tested whether iron and E2 together enhance greater cell proliferation than either alone. MCF-7 cells were treated with E2 and/or iron in α-MEM media containing 0.1% FBS (to minimize transferrin iron in serum, a confounding factor). Our results have shown that E2 and iron have induced higher cell proliferation (15.3-fold of control) than E_2 (6.1-fold) or iron (7.9-fold) alone. Western blot of E2 treated cells showed that E_2 also induced transferrin receptor, a membrane protein involved in cellular iron uptake, in ER^+ cells but not in ER^- cells. In conclusion, our results indicate that E2 is most likely the effective compound in HRT that induces proliferation of only ER^+ breast cells. Whether iron is an inter-dependent growth factor in E2-induced ER^+ breast cell growth awaits further investigation. [This work was supported by DOD grant # DAMD 17-03-01-0717]

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1133 Iron metabolism and oxygen tension: effect on hypoxia inducible factor (HIF) stabilization

Jonathan Eckard, Krystyna Frenkel, Xi Huang. *NYU School of Medicine, New York, NY.*

Iron is an element essential for functions of many enzymes as well as DNA synthesis in the human body. It has long been suspected that iron contributes to cancer development by oxidative DNA and protein damage, which therefore has been the focus of cancer research related to this metal. Recent studies indicate that iron is an important co-factor of HIF-prolyl hydroxylase, an enzyme involved in HIF degradation. HIF is the most recognized for controlling the regulation of an array of genes helping cells adapt to low oxygen conditions. The goal of this research has been to examine how cellular iron participates in oxygen sensing and cellular hypoxic response. We hypothesized changes in cellular iron levels during hypoxia contribute to HIF stabilization and downstream biological events. For the experiments in this study, we have used MDA-MB-231 human breast carcinoma cells as our model. We have shown that cells exposed to 1% O_2 for 6 h responded with significant decreases in cellular iron bound to low molecular weight chelators (LMW-Fe;81.9 *vs.* 31 nmol/mg protein; $p<0.04$) as well as in ferritin (Ftn), an iron storage protein, (20.1 *vs*. 9.5 ng/mg total protein, $p<0.05$), as compared to normoxia. Our results have also shown that, when compared to normoxic controls, hypoxic exposure increased iron regulatory protein (IRP) binding suggesting that cells are in an iron-depleted state during hypoxia. To corroborate this observation, cells were treated with iron prior to hypoxia. We have found that iron pretreatment results in increased levels of LMW-Fe (1.98 *vs*. 0.93 nmol/mg protein, $p<0.01$) concomitant with decreased HIF protein levels, as detected by Western blotting (densitometer readings -82% of control). Cumulatively, these results suggest that elevated cellular iron can cause HIF destabilization and could further implicate iron as playing a role in HIF-prolyl hydroxylase activity. Currently, we are examining the effect of decreased iron levels on HIF stabilization and down stream biological effects through blocking the expression of transferrin receptor. [This work was supported in part by grant DOD grant # DAMD 17-03-01-0717, NIEHS grant ES00260, and Superfund ES10344]

Citation format: Proc Amer Assoc Cancer Res 2005;46:1133.

Cancer Res.

Enhancement of cell proliferation by iron and estrogen in breast epithelial cells with different status of estrogen and progesterone receptors– A role for iron in breast cancer of post-menopausal women?

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Running title: Role of iron in breast cancer

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Abstract:

For many years it has been known that estrogen as well as hormone replacement therapy (HRT) increase the risk of breast cancer in post-menopausal women. Due to the cessation of menstrual bleeding, this population generally has a high level of iron. In the present study, we have investigated whether estrogen and iron synergistically enhance proliferation of various cells with different status of estrogen receptor (ER) and progesterone receptor (PR). Human breast cancer cell line, MCF-7 (ER^+) and non-cancerous breast epithelial cell line MCF-10A (ER^-), as well as two murine mammary cancer cell lines, one MXT^+ (ER^+/PR^+) and the other MXT^- (ER^-/PR^+) were used. Crystal violet assay was employed to determine cell proliferation. Our results have shown that Prempro^{TM} (Wyeth-Ayerst Pharmaceuticals), the most prescribed HRT drug, have induced cell proliferation only in ER^+ cells. For example, at 10 $\mu g/cm^2$ of dose, Prempro-treated MCF-7 cell proliferation was 2.3-fold of control in α-MEM completed with 10% fetal bovine serum (FBS). To further distinguish the effects of estradiol (E2) and progesterone (Pg) on cell proliferation, MCF-7 cells were separately treated with E2 ($1x10^{-9}$ M) or Pg ($4x10^{-9}$ M). Results showed that E2 is the main component in inducing cell proliferation. MCF-7 cells were then treated with E2 and/or iron in α-MEM media containing 0.1% FBS (to minimize transferrin iron in serum, a confounding factor). Our results have shown that E2 and iron have induced higher cell proliferation (15.3-fold of control) than E_2 (6.1-fold) or iron (7.9-fold) alone. Western blotting of E2-treated cells showed that E_2 also induced transferrin receptor (TfR), a membrane protein involved in cellular iron uptake. In conclusion, our results suggest that iron and estrogen may be concomitantly involved in the cell proliferation of ER^+ breast cells and together may contribute to the increased risk of breast cancer in post-menopausal women.

Key words: Iron, estrogen, breast cancer, hormone replacement therapy, progesterone, tamoxifen, estrogen receptor.

Abbreviations: α-MEM: alpha-minimal essential medium; ER: estrogen receptor; FBS: fetal bovine serum; HPO: hypothalamic-pituitary-ovarian; HRT: hormone replacement therapy; PR: progesterone receptor; Tam: tamoxifen; TfR: transferrin receptor.

Introduction:

Breast cancer incidence rates are higher in post-menopausal women than in pre-menopausal women. The most recent data from the American Cancer Society showed that, since 1986, breast cancer incidence rates have increased only in post-menopausal women aged 50 and older (http://www.cancer.org). Several of the well-established risk factors for breast cancer, such as early age at menarche, nulliparity, late first full-time pregnancy, and/or late menopause, have suggested that a long life-time exposure to estrogens contributes to breast cancer development (1). A meta-analysis of nine prospective studies on post-menopausal levels of endogenous sex hormones and breast cancer showed a strong association of estrogens with breast cancer risk (2). Recent epidemiological studies from the Women's Health Initiative indicate that hormone replacement therapy (HRT) may also increase the risk of breast cancer in post-menopausal women (3).

Menopause is a natural aging process during which time a woman passes from the reproductive to the non-reproductive years. During the natural menopause transition, the dynamics of the hypothalamic-pituitary-ovarian hormones change dramatically from cyclic to static pattern and levels of estrogen and progesterone from high to low. Although estrogen is no longer solely an endocrine factor in post-menopausal women, estrogen is produced locally in breast tissue by aromatase cytochrome P450 (P450arom, the product of the CYP19 gene) (4-6). Despite the decline in serum estrogen levels after menopause, breast tissue E2 levels in pre- and post-menopausal women do not differ significantly. The prostaglandin E2 increases intracellular cAMP levels and stimulates estrogen biosynthesis as a consequence of overexpression of the cyclooxygenase type II (COX-2) (7, 8). These observations of low serum level and comparable breast tissue level of estrogen but a high breast cancer rate strongly suggest that factors other

than estrogens may contribute to the greater breast cancer incidence in post-menopausal women. Because of the female baby-boomer generation entering into menopause, there is an urgent need to addresses additional breast cancer etiology factors in this high risk population.

One of the most important physiological differences between pre- and post-menopausal women is in iron levels. Due to the cessation of menstrual bleeding, serum levels of iron, such as ferritin and serum iron, also known as transferrin iron, are much higher in post-menopausal women than in pre-menopausal women (9-11). Ferritin is an iron storage protein with a capacity of binding up to 4500 atoms of iron per molecule of ferritin (12). Transferrin, an iron transport protein with two binding sites for iron, tends to be more saturated in post-menopausal women than in pre-menopausal women (13, 14). Therefore, iron may be an important etiological factor in the development of breast cancer in post-menopausal women. In the present study, we investigated the effects of Prempro^{TM}, the most prescribed drug in the United States for post-menopausal symptoms, as well as estradiol (E2), progesterone (Pg), and iron on cell proliferation. Various cell lines with different status of estrogen receptor (ER) and progesterone receptor (PR) were used in an *in vitro* tissue culture system. Our results suggest that high levels of iron, due to the cessation of menstrual blood, in conjunction with HRT or endogenous high levels of estrogen, may contribute to breast cancer development in post-menopausal women.

Materials and methods:

Chemical reagents:

Fetal bovine serum (FBS), antibiotics, and L-glutamine were obtained from Atlanta Biologicals (Norcross, GA). Alpha minimum essential medium (α-MEM), tamoxifen (Tam), 17β–estradiol (E2)-water soluble, progesterone (Pg), ferrous sulfate septahydrate, crystal violet, glutaraldehyde, ethanol, and monoclonal mouse-anti-β-tubulin antibodies were purchased from

Sigma-Aldrich (St. Louis, MO). Human soluble transferrin receptor (sTfR) was from R & D System (Minneapolis, MN). Other reagents were: mouse-anti-TfR antibodies (Research Diagnostics Inc., Flanders, NJ), peroxidase-conjugated anti-mouse antibody (Cell Signaling, Beverly, MA), cell lysis M-Per buffer (Pierce, Rockford, IL), Prempo^{TM} 0.625mg/2.5 mg tablet containing 0.625 mg conjugated estrogens and 2.5 mg of medroxy-progesterone acetate (Wyeth-Ayerst Pharmaceuticals, Philadelphia, PA).

Cell culture:

Human breast cancer MCF-7 cell line and the immortalized human breast epithelial MCF-10A cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA). MXT^+ cell line was derived from the murine mammary cancer model MXT-M-3,2 MC (hormone sensitive), which was first induced by urethane treatment in female C57BLxDBAfF1 mice as previously described (15). MXT^- cell line was derived from the MXT-M-3,2 (OVX) MC (hormone insensitive) mice (16). MXT^+ cells with estrogen receptor positive and progesterone receptor positive (ER^+/PR^+) and MXT^- cells (ER^-/PR^+) were previously characterized (17). All cells were cultured in α-MEM containing 10% FBS and 0.9 mg/ml L-glutamine in a humidified atmosphere of 95% air, 5% CO_2 at 37^{o}C. Figure 1 shows the ER status of the four cell lines used, which confirmed their differences in ER levels and, thus, provide a useful tool for the present study.

Crystal Violet Assay for Cell Proliferation:

To evaluate cell proliferation, crystal violet assay was used as previously described (17). In brief, cells were seeded in a 96-well plate containing 100 μl complete α-MEM per well with 12 cells per microscopic field (400 X). After 48h incubation, cell culture medium was replaced with fresh α-MEM at 200 μl/well. To test the effect of Prempro^{TM}, the tablet was ground in an

agar mortar and suspended in water. The freshly prepared suspensions were immediately added into each well for cell treatment. Data were presented as $\mu g/cm^2$, with 1 $\mu g/cm^2$ equivalent to 10^{-9} M E2 assuming that the conjugated estrogens are completely solubilized. E2, Pg, Tam, and iron as ferrous sulfate were all freshly prepared before cell treatment. For the effect of Tam, Tam was added 2 h before E2 treatment. For the combined effects of iron and E2, both chemicals were added simultaneously.

For each concentration, sixteen replicates (wells) were used in one microplate. One microplate was also used for each time point (0 h, 48 h, 72 h, 96 h and 120 h). After treatments at various times and concentrations, the cell culture medium was removed. One hundred μl of 1% (w/w) glutaraldehyde dissolved in 1x PBS were then added into each well and incubated for 15 min at room temperature. The glutaraldehyde solutions were subsequently replaced with 150 μl 1x PBS and the plate was stored at 4^oC until the last one was collected. Cells in each well were then stained with 0.02% crystal violet aqueous solution (100 μl/well) for 30 min. After washing off the excess dye with water, cells in each well were re-dissolved in 70% ethanol at 180 μl/well. After shaking on a plate shaker for 3 h, absorbance was recorded at 578 nm on a microplate reader (UV-Vis, Molecular Device, CA).

Effects of chemical reagents on cell proliferation were calculated as follows (17): [%] $T/C = (T-C_0)/(C-C_0) \times 100$ with T representing the mean absorbance of the treated cells, C representing the mean absorbance of the controls, and C_0 representing the mean absorbance of the cells at time zero.

Determination of TfR by Western blotting:

To investigate the effects of E2 and/or iron on TfR expression, MCF-7 cells were treated as follows: Cells were initially grown in a 10-cm Petri dish containing α-MEM completed with

10% FBS. Some cells were then treated with E2 at 5 nM or 10 nM for 24 h, and the remaining cells were used as control or for subsequent iron treatment. After E2 treatment, all cells were washed with PBS and media were changed to 0.1% FBS α-MEM in order to minimize levels of transferrin iron in serum, a confounding factor. Iron was then added to the dishes at 25 μM or 50 μM for 24 h. After treatment, culture media were collected for measurements of TfR and cells were collected using a rubber policeman, lysed with M-Per lysis buffer (Pierce). Cell lysates (30 μg protein) were subjected to 12% SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 and probed with antibodies against TfR (1:500) or β-tubulin (1:3000). Antibody banding signals were visualized with peroxidase-conjugated anti-mouse antibody using Western Lightning Plus Chemiluminescence Reagent (Perkin-Elmer).

Measurements of TfR by ELISA:

TfR in tissue culture media was determined by ELISA using two different monoclonal antibodies specific for TfR (R & D System, Minneapolis, MN). In brief, 20 μl culture media or standards were pipetted into wells of a microplate pre-coated with a monoclonal antibody that can capture TfR, thereby immobilizing TfR to the well. After washing away any unbound protein, an anti-TfR monoclonal antibody conjugated to horseradish peroxidase was added. After washing away unbound conjugated antibody, the amount of conjugate remaining in the well is proportional to the amount of TfR initially captured. The amount of conjugated enzyme was measured by incubation with a chromogenic substrate at 450 nm.

Statistical analysis:

The experimental differences were determined by two-tailed Student's *t*-test. To assure reproducibility, the experiments were repeated at least three times. Graphed data represent the

means ± SE. A confidence level of $p<0.05$ was taken to represent a significant difference in all cases.

Results:

Effects of Prempro™ on cell proliferation of mouse mammary cancer MXT cells:

Prempro™ is the mostly prescribed drug for HRT in the United States (18). Figure 2 shows that Prempro™ increases cell proliferation in MXT^+ cells, which are ER^+ and PR^+. Interestingly, Prempro™ had no effects on cell proliferation of MXT^- cells, which are ER^- but PR^+. The increases in cell proliferation were Prempro™ dose-dependent at 24 h treatment. After 48 h, cell proliferation of MXT^+ cells reached a maximal growth level in the 96-well microplate with 10% FBS-containing α-MEM. Therefore, no significant differences were observed between the control MXT^+ and the Prempro™–treated groups (data not shown). However, MXT^- cells treated with Prempro™ at 10 $\mu g/cm^2$ for 72 h or longer showed an inhibition as compared to the control MXT^- cells (73.4 $\pm$ 1.1% of control readings). Since cellular PR levels were similar between MXT^+ and MXT^- cells, these results indicate that difference in ER status between the two cell lines is the main factor responsible for the difference in cell proliferation induced by Prempro™ treatment.

Effects of Prempro™ on cell proliferation of human breast cells with different ER status:

To further confirm the above observations, human breast cancer cells MCF-7 (ER^+) and non-cancerous breast epithelial cells MCF-10A (ER^-) were used for Prempro™ treatment. Figure 3 shows that cell proliferation increased in ER^+ MCF-7 cells but not in ER^- MCF 10A cells. Similarly to MXT^+ cells, the increase is Prempro™ dose-dependent, although the maximal growth time was 48 h. There was no increase in cell proliferation in ER^- MCF-10A cells treated with low doses of Prempro™. In fact, at high doses ($\geq$ 5 $\mu g/cm^2$), Prempro™ inhibited ER^- cell

growth (data not shown). Because of the ER^+ status of MCF-7 cells, this cell line was chosen for the subsequent studies.

Effects of E2, Pg, and Tam on cell proliferation of MCF-7 cells:

MCF-7 cells were separately treated with 1 nM E2 or 4 nM Pg and/or 5 μM Tam. Figure 4 shows that E2 significantly increased cell proliferation by 289% over that of the control cells after 72 h treatment. After 48 h treatment, the increase over the control was about 102% for 1 nM E2 and 123% for 10 nM E2, results similar to 48 h treatment with Prempro^{TM} at 10 μg/cm^2 (Fig. 2). As expected, Tam, an antagonist for ER, significantly inhibited E2-induced cell proliferation, although the growth of MCF-7 cells pre-treated with Tam followed by E2 was significantly higher than that of the control MCF-7 cells (97% higher). Surprisingly, pure Pg also significantly increased MCF-7 cell proliferation (89% higher than the control cells). These results are different from the MXT^- cells (ER^-/PR^+) treated with Prempro^{TM}, which showed no effects on MXT^- cell proliferation. These results suggest that medroxy-progesterone acetate in the Prempro^{TM} tablets may not enhance cell proliferation or PR in the MXT^- is not functional. Tam itself had no effect on MCF-7 or on Pg-induced cell proliferation.

Additive or synergistic effects of E2 and iron on cell proliferation:

One of the physiological differences between pre- and post-menopausal women is that, in pre-menopausal women, iron stores remain low due to loss of menstrual blood (10, 14). In post-menopausal women, iron levels dramatically increase due to the cessation of the menstrual bleeding. The second goal of this exploratory study was to determine whether iron contributes to E2-mediated cell proliferation. MCF-7 cells were treated with E2 and /or iron. Figure 5 shows that E2 or iron alone significantly increased MCF-7 cell proliferation as compared to the same cells without any treatment. Interestingly, E2 and iron together enhanced cell proliferation more

than either alone. Though the effects appear additive, this is an important observation due to the high levels of iron in post-menopausal women.

Up-regulation of TfR by E2:

To further investigate whether E2 interact with iron on cell proliferation, Western blotting and ELISA techniques were employed to detect transferrin receptor (TfR), a dimeric membrane protein with molecular weight of 90 kDa controlling cellular iron uptake (19, 20). Figure 6 showed that iron at 25 and 50 μM down-regulated the expression of TfR protein in MCF-7 cells (lanes 2 and 3), a normal iron metabolism through a negative feed back mechanism. Most interestingly, E2 at 5 nM and 10 nM induced TfR (lanes 4 and 7). This induction may be due to the increased cell proliferation, which requires more iron at the time when iron supply is limited. However, when iron is present in the tissue culture medium, the induction of TfR by E2 is not significant (lanes 5, 6, and 8). To further confirm the result of Western blotting, Figure 7 shows that iron significantly decreased culture media levels of TfR while E2 increased TfR levels, results paralleling those of the Western blotting. These results suggest that the membrane-bound TfR can be released into the culture medium.

Discussion:

Current breast cancer research focuses mainly on hormones and their receptors. Results of our study suggest that iron may play an important role in breast cancer of post-menopausal women. The roles of iron and estrogen in cancer have long been suspected but not specifically studied in tandem, particularly in relation to breast cancer (21, 22). Estrogens influence the growth, differentiation, and function of tissues of the female reproductive system, *i.e.*, uterus, ovary, and breast (23). However, estrogen's growth-stimulating effects could make it a cancer promoter (24, 25). New work suggests that products estrogen forms in the body may also cause

the initiating mutations (26, 27). 4-hydroxyestrone (4-HE), 16α-hydroxyestrone (16-α), and 4-hydroxyequilenin are the main metabolites that may undergo alkylation and oxidation of DNA triggering cancer development (27-29). The effects of estrogens in the breast tissue are mediated by ER (30). ERα and ERβ are expressed in normal as well as malignant tissues. A high level of ERα expression is characteristic of breast carcinomas (30). In clinical practice, ER is analyzed simultaneously with PR in breast cancer biopsies. Since the expression of PR is induced by estrogens *via* the ER, the presence of both receptors indicates a functional ER.

In the present study, we began with two mouse mammary cancer cell lines MXT^+ and MXT^- with comparable levels of PR but different ER status. Proliferation assay clearly showed that Prempro^{TM}, the drug used for HRT in post-menopausal women, enhances cell proliferation only in MXT^+ cells, which are ER^+ and PR^+. In MXT^- cells, which are ER^- and PR^+, Prempro^{TM} had no effect on cell proliferation. Similarly, in human breast cells, only ER^+ MCF-7 cells responded favorably to Prempro^{TM} –induced cell proliferation. These results suggest that equine estrogen in the tablet may be the main active ingredient causing cell proliferation. Because Prempro^{TM} had no cell proliferating effect on MXT^- (ER^-/PR^+) or MCF-10A (ER^-) cells, these results also suggest that medroxy-progesterone in the tablet is inactive in stimulating cell growth or the PR in the MXT^- cells is not functional (Figures 2 and 3). These results are different from Figure 4, which shows that pure Pg significantly enhanced MCF-7 cell proliferation, whereas Tam, an E2 antagonist, decreased E2- and Pg-induced proliferation of the same cells.

Iron, like estrogens, is essential for cell growth and metabolic processes that include oxygen transport, enzyme functions, DNA synthesis, and electron transport (31). Yet, mounting evidence indicates that increased iron levels in the body may be associated with increased risk of cancer (32). During menopause or perimenopause, iron accumulates slowly and is chelated by

iron proteins such as ferritin and transferrin. Our understanding is that, under pre-menopausal conditions, iron status leans towards iron deficiency or slightly anemic conditions. Therefore, iron in the pre-menopausal women is not readily available for adverse health effects. However, under post-menopausal conditions, iron starts to accumulate due to the cessation of menstrual bleeding and iron status leans towards iron overload. Under these circumstances, total iron levels are higher in post-menopausal women than in pre-menopausal women. Investigation into whether combined iron and E2 enhance cell proliferation showed an additive effect of E2 and iron on the growth of MCF-7 cells (Figure 5).

These results lead us to determine whether iron and E2 interact. It has been known for a long time that the cell uptake of transferrin-bound iron depends on the number of membrane TfR (19, 33). Increases in intracellular iron down-regulate the TfR numbers and, thus, lead to a decreased iron uptake (34, 35). As expected, cells treated with iron down-regulated TfR expression (Figure 6). Surprisingly, E2 up-regulated TfR expression in a dose-dependent manner, as revealed by Western blotting as well as by ELISA of tissue culture media (Figure 7). Previous studies have shown that E2 can induce a membrane glycoprotein, which is a novel transferrin binding protein, structurally related to the stress-regulated proteins in MCF-7 cells and lactoferrin in the female reproductive tract of mouse, rat, and hamster (36, 37). A recent study has further demonstrated that E2 induces transferrin gene expression in MCF-7 cells through a nonconsensus distal estrogen responsive element (38). In our study, it seems that this E2-induced TfR up-regulation is iron-independent. It has been shown that membrane TfR levels are higher in the breast cancer cells than in normal breast epithelial cells (39-41). These results strongly suggest that increased E2 levels in breast tissue may increase membrane TfR levels and, thus, enhance iron uptake for cell growth.

In conclusion, our results suggest that iron and estrogen may be concomitantly involved in the cell proliferation of ER^+ breast cancer cells. Further advances in understanding the roles of estrogen, iron, and their receptors in breast cancer should greatly benefit the health of women, who are entering into menopause and are at greatly increased risk of developing breast cancer. For example, identification of iron as a risk factor in breast cancer etiology may lead to a potential new clinical therapy that targets TfR by a mechanism akin to tamoxifen blocking ER.

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Legends:

Figure 1. ER status of different cell lines. ER was analyzed by Western blot. Beta-tubulin was used as an internal control for protein loading.

Figure 2: Effects of Prempro^{TM} on cell proliferation of mouse mammary cancer MXT^{+} but not MXT^{-} cells. MXT^{+} (ER^{+}/PR^{+}) and MXT^{-} (ER^{-}/PR^{+}) cells were treated with 0, 1, 2, 5 and 10 μg/cm^2 of Prempro^{TM} in 10% FBS, 0.9 mg/ml L-glutamine, and antibiotics-free α-MEM medium in a 96-well plate for 24 h. *: Significantly different from control, $p<0.05$.

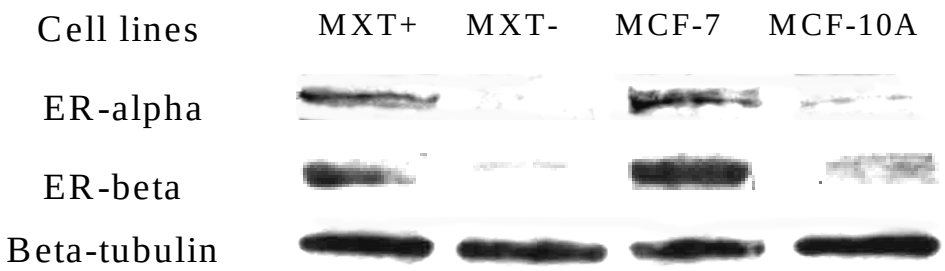
Figure 3: Effects of Prempro^{TM} on cell proliferation of human breast cancer MCF-7 but not MCF-10A cells. MCF-7 (ER^{+}) and MCF-10A (ER^{-}) cells were treated with 0, 1, 2, 5 and 10 μg/cm^2 of Prempro^{TM} in 10% FBS α-MEM medium in a 96-well plate for 48 h. *: Significantly different from control, $p<0.05$.

Figure 4: Effects of estradiol (E2), progesterone (Pg), and/or tamoxifen (Tam) on cell proliferation of human breast cancer MCF-7 cells. MCF-7 cells were treated with E2 at 10^{-9} M, Pg at $4x10^{-9}$ M, and Tam at 5 μM for 72 h. The ratio of E2**:** Pg is1**:**4, equivalent to the same ratio as in Prempro^{TM}. *: Significantly different from control, $p<0.05$. &: Significantly different from cells treated with E2 or Pg alone, $p<0.05$.

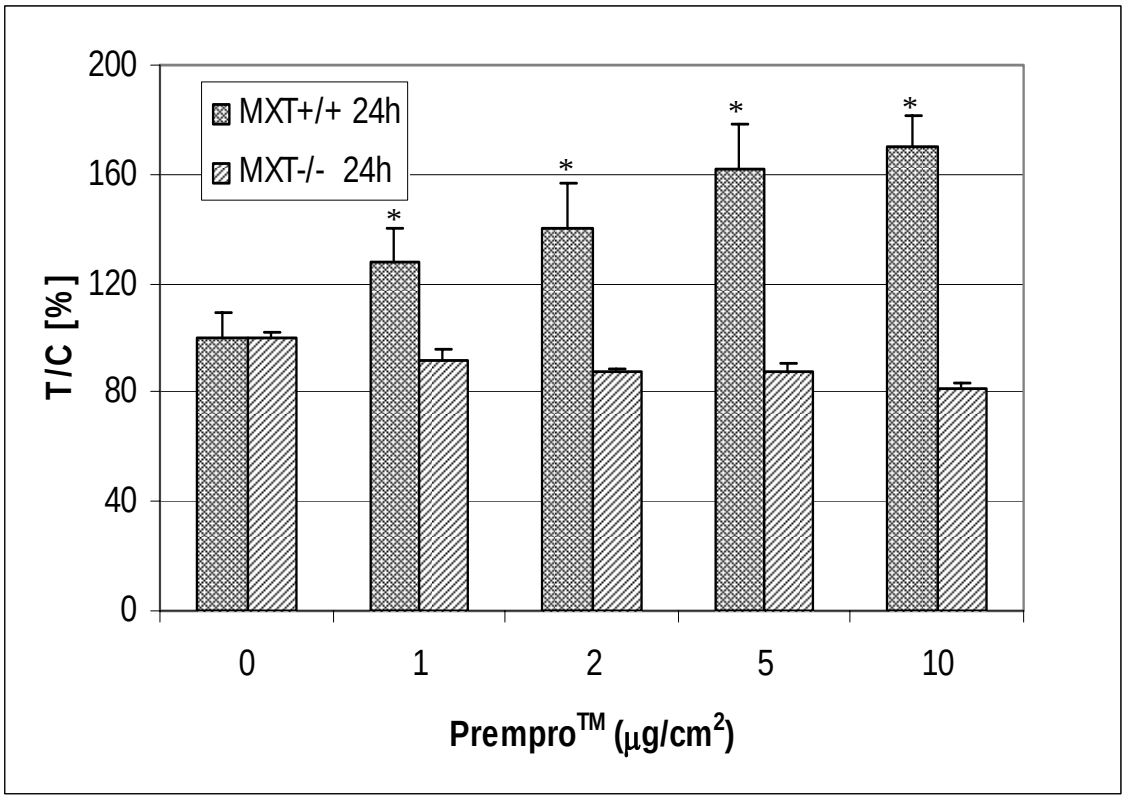
Figure 5: Additive effects of E2 and iron on MCF-7 cell proliferation. MCF-7 cells were treated with $1x10^{-9}$ M E2and/or 10 μM Fe in 0.1% FBS α-MEM for 24 h. E2 and Fe together significantly increased cell proliferation as compared to E2 or Fe treated cells alone. *: Significantly different from control, $p<0.05$. &: Significantly different from cells treated with E2 or iron alone, $p<0.05$.

Figure 6: Enhancing effects of E2 and inhibitory effects of iron on TfR regulation in MCF-7 cells. Cells were treated with E2 at $5x10^{-9}$ M (+) and $10x10^{-9}$ M (++) in a 10-cm cell culture dish in 10% FBS α-MEM for 24 h, medium removed, and then followed by iron at 25 μM (+) and 50 μM (++) in 0.1% FBS α-MEM for additional 24 h. Cells were collected and lysed using M-Per lysis buffer. Thirty μg of protein samples were loaded to each well for TfR and β-tubulin Western blotting.

Figure 7: Effects of E2 and/or iron on TfR levels in tissue culture media. MCF-7 cells were treated with E2 and/or iron as described in Fig. 6. Cell culture media were collected for soluble transferrin receptor (TfR) determination using ELISA method. *: Significantly different from control, $p<0.05$.



Dai et al., Figure 1



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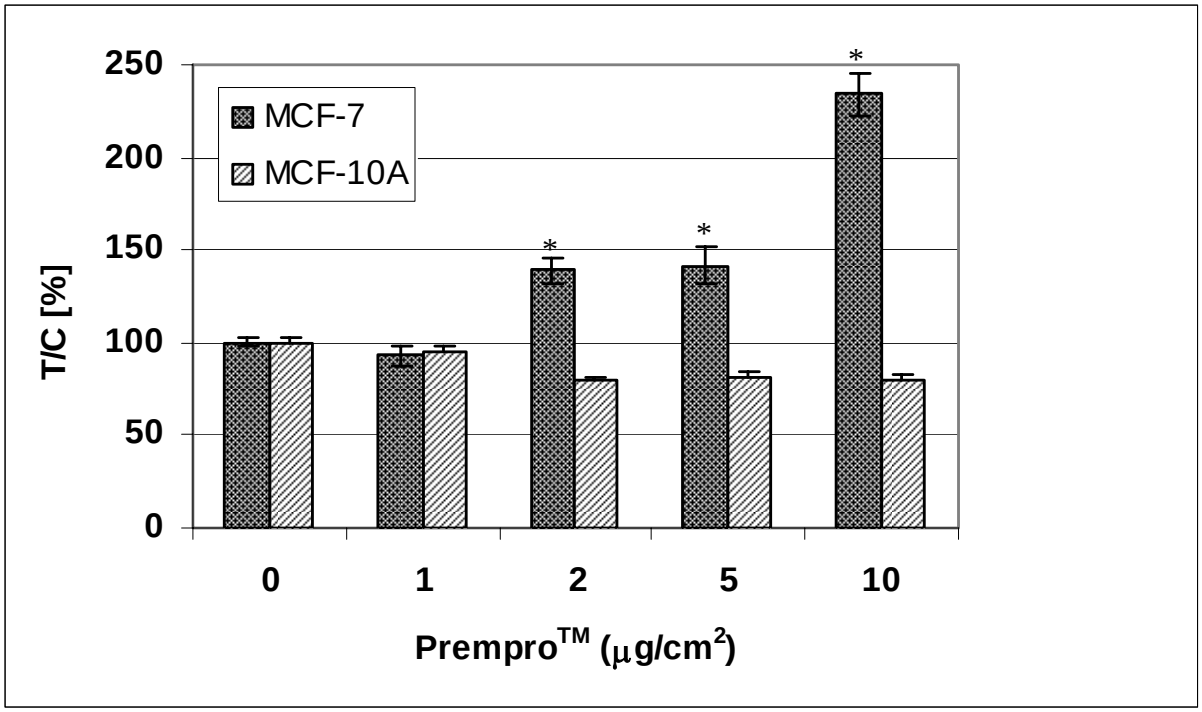

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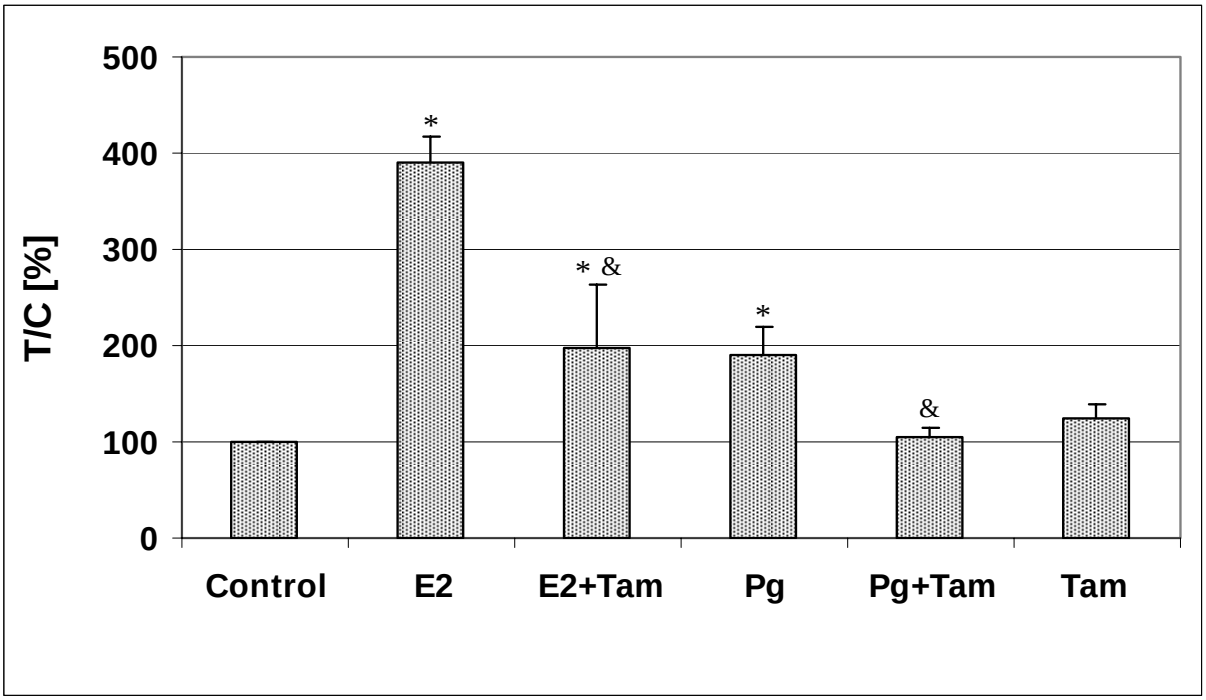
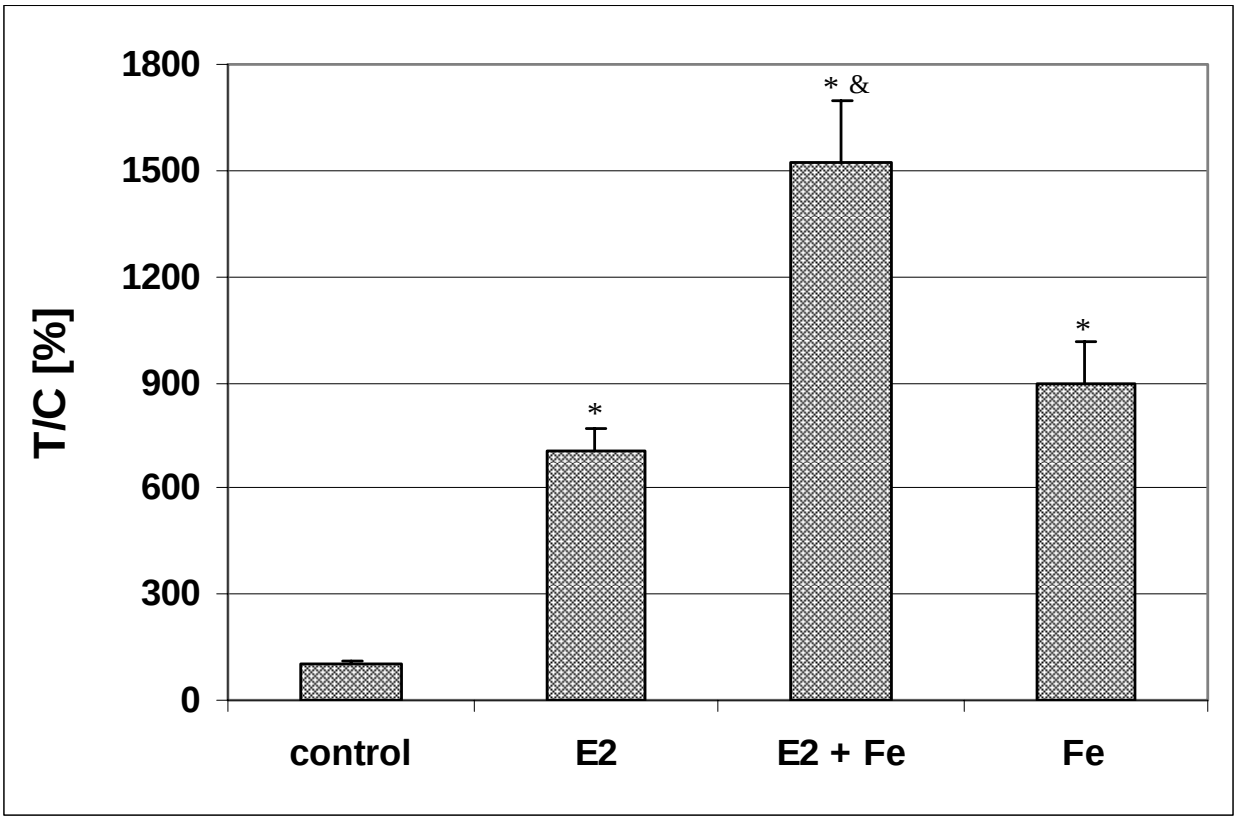
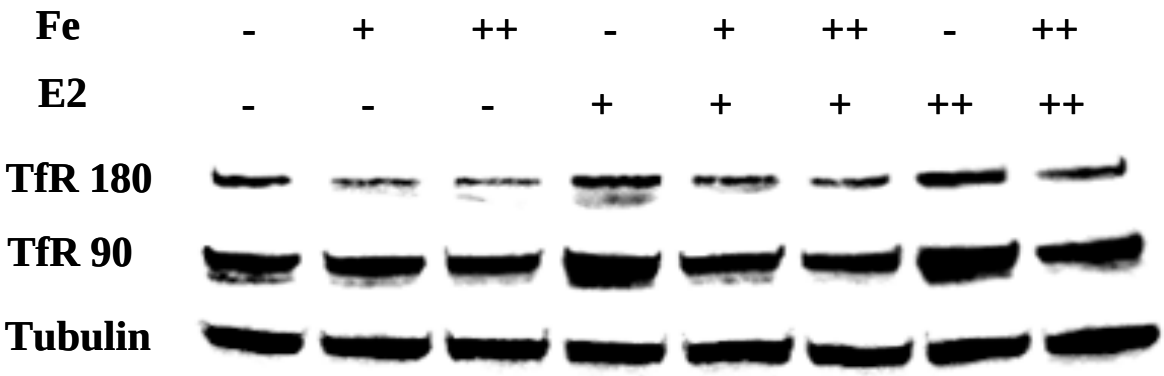


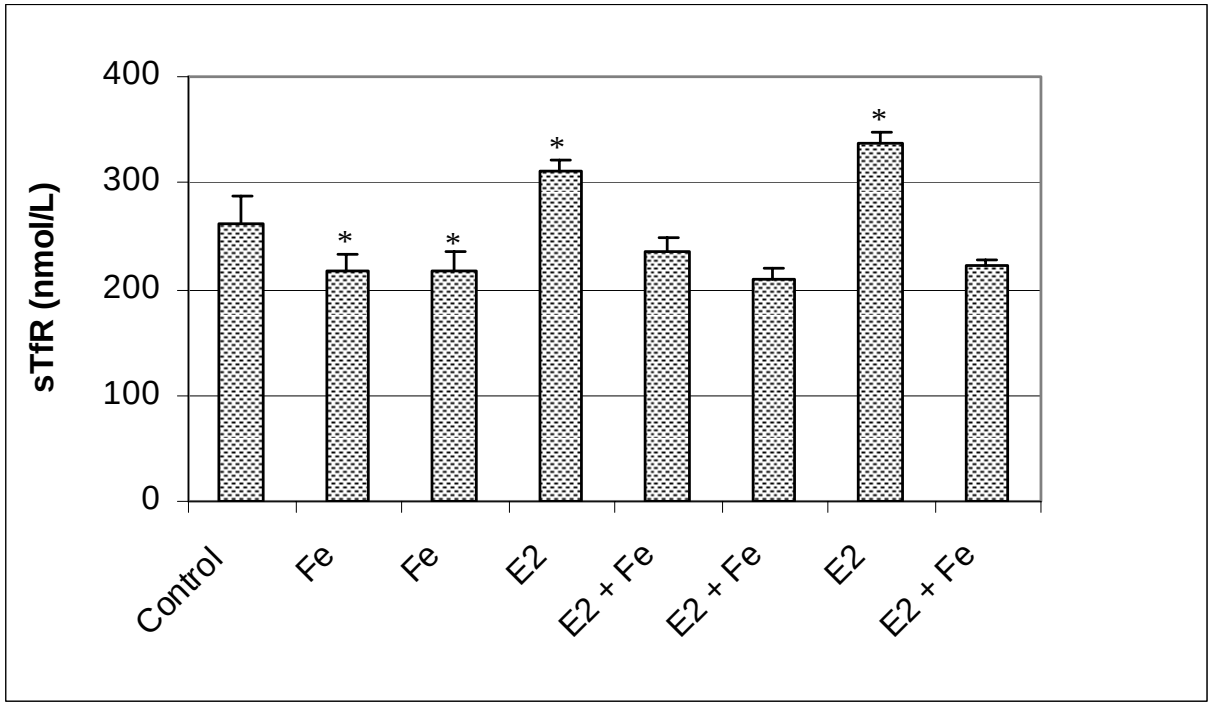
Dai et al., Figure 4



Dai et al., Figure 5



Dai et al., Figure 6



Dai et al., Figure 7